

Proposal for combining *Bradyrhizobium* spp. (*Aeschynomene indica*) with *Blastobacter denitrificans* and to transfer *Blastobacter denitrificans* (Hirsch and Muller, 1985) to the genus *Bradyrhizobium* as *Bradyrhizobium denitrificans* (comb. nov.)

Peter van Berkum^{a,*}, Juanita M. Leibold^b, Bertrand D. Eardly^b

^aSoybean Genomics and Improvement Laboratory, Agricultural Research Service, US Department of Agriculture, HH-4, Bldg 010, BARC west, 10300, Baltimore Blvd., Beltsville, MD 20705, USA

^bPenn State Berks-Lehigh Valley College, Berks Campus, P.O. Box 7009 Reading, PA 19610, USA

Received 27 April 2005

Abstract

The symbiotic bradyrhizobia of *Aeschynomene indica* and the aquatic budding bacterium *Blastobacter denitrificans* have much in common and this study broadens the characters that are shared between the two. The 23S rRNA gene sequences of the bradyrhizobial isolates were most similar to each other and to the sequence of *Bl. denitrificans*. Evidence for the presence of photosynthetic genes in the genome of *Bl. denitrificans* was obtained by PCR using primers to the conserved M subunit (*pufM*) of the photosynthetic reaction center present in purple sulfur and purple nonsulfur bacteria. The deduced amino acid sequences of the partial PufM protein of *Bl. denitrificans* and the corresponding sequences obtained from the bradyrhizobial isolates were identical. Both the bradyrhizobial isolates and the type strain of *Bl. denitrificans* shared the ability to propagate by budding, demonstrated by electron microscopy. Even though many interspecific characters were shared among the bradyrhizobial isolates including *Bl. denitrificans*, it was evident from Amplified Fragment Length Polymorphism (AFLP) analysis that genomic variation existed among the collection that was examined. Variation among bradyrhizobial isolates and *Bl. denitrificans* also was established in carbon and nitrogen source utilization and the ability to grow at elevated temperature. Based on these results and previously reported evidence it is suggested that the type strain for *Bl. denitrificans* and the bradyrhizobial isolates from nodules of *A. indica* belong to a common group of bacteria. Therefore, it is proposed that they be combined into the genus *Bradyrhizobium* and that LMG 8443 be transferred to this genus as the type strain for *B. denitrificans*.

© 2005 Elsevier GmbH. All rights reserved.

Keywords: *Aeschynomene indica*; *Bradyrhizobium*; *Blastobacter*; Symbiosis; Nodulation; Nitrogen fixation; Phototroph; 16S; 23S; PufM

Introduction

Soil bacteria collectively referred to as rhizobia form a symbiotic relationship with leguminous plants. This symbiosis is evident by the presence of hypertrophies on

*Corresponding author. Tel.: +1 301 504 7280;
fax: +1 301 713 0308.

E-mail address: pberkum@ba.ars.usda.gov (P. van Berkum).

the host plant roots and by the benefit the plant derives from bacterial nitrogen fixation. In agriculture, this property is exploited by management of the symbiosis for enhancing efficiency in legume crop production. Management of the symbiosis is necessary because there is specificity in the interaction between plant and microbe. Also, the appropriate bacteria for a specific leguminous crop may be absent from soils in production fields.

The rhizobia initially were classified in the single genus *Rhizobium* until Jordan [10] suggested their taxonomic separation based on their growth rate in pure culture to distinguish between the “fast-growing” and the “slow-growing” strains. The genus name *Bradyrhizobium* was proposed for all “slow-growing” strains and at that time included only one species, *Bradyrhizobium japonicum*, the symbiont of soybean. Other strains of *Bradyrhizobium* are symbionts of a diverse range of legumes that includes the genus *Aeschynomene*.

Within the genus *Aeschynomene* some species, such as *Aeschynomene indica*, are unusual because they may form hypertrophies on branches and stems as well as the roots. In addition, the symbiotic bacteria of *A. indica* and some other species of this leguminous plant are unique because some, but not all, produce photosynthetic pigments [2,4,5,31]. Based on these and some other phenotypic characters, Eaglesham et al. [4] proposed that these photosynthetic symbiotic bacteria be classified as *Photorhizobium thompsonianum* with BTAi1 [3] as the type strain. However, based on high 16S rRNA gene sequence similarity with *B. japonicum* it was more acceptable that they be classified as undefined species of *Bradyrhizobium* [16,36,37]. Also, based on high 16S rRNA gene sequence similarity, the aquatic budding bacterium *Blastobacter denitrificans* was placed within the same group [34].

Zavarzin [38] originally proposed the genus *Blastobacter* and described the type species, *Bl. henricii*, from cells observed in lake water. However, the type strain for this species was not isolated in pure culture. Subsequently, four additional cultures were obtained from aquatic habitats and were chosen to represent four additional species, *Bl. aggregatus*, *Bl. capsulatus*, *Bl. denitrificans*, and *Bl. natatorius* [23,26]. Each of these species epithets has been listed on the Approved Lists of Bacterial Names [17].

The genus *Blastobacter* is highly heterogeneous since the described species are placed into at least three distinct branches of the α -subdivision of the *Proteobacteria* according to 16S rRNA gene sequence variation [9]. Consequently, Hugenholtz et al. [9] suggested that, with the exception of *Bl. aggregatus* and *Bl. capsulatus*, the other described species should be reclassified as separate genera. As a solution Sly and Cahill [24] proposed that new genera be created for the existing

validated species, and that the type strain for *Bl. natatorius* be transferred to the new genus *Blastomonas*. The type strain for this genus was named *Blastomonas natatoria*.

Recently, we reported high sequence similarity of the Internally Transcribed Space (ITS) region between the 16S and 23S rRNA genes of *Bl. denitrificans* with BTAi1 and 15 additional bradyrhizobial isolates of *A. indica*. From subsequent plant inoculation trials in the greenhouse it was shown that *Bl. denitrificans* can nodulate *A. indica* and is capable of forming a nitrogen-fixing symbiosis with this legume [28]. The ability to form a nitrogen-fixing symbiosis with legumes is a common characteristic of most, but not all genera within the order Rhizobiales. Considering the suggestion by Hugenholtz et al. [9] and the symbiotic nitrogen-fixing capability of *Bl. denitrificans* we decided to further investigate the commonality between *Bl. denitrificans* and the bradyrhizobia that were isolated from *A. indica*. Based on the results of our investigation we propose that *Bl. denitrificans* be transferred to the genus *Bradyrhizobium* and that this species be combined with the bradyrhizobial isolates of *A. indica* to form the new species *Bradyrhizobium denitrificans*.

Materials and methods

Bacterial strains

The strains used in this study were obtained from the USDA ARS National *Rhizobium* Germplasm Collection, were originally isolated by van Berkum et al. [31]. *Bl. denitrificans* type strain IFAM 1005 (LMG 8443) was kindly provided by the Belgian Culture Collection of Microorganisms. The sources of *A. indica* strain BTAi1, *Methylobacterium extorquens* ATCC 8457 and ATCC 14718, *M. organophyllum* ATCC 27886, *M. rhodinum* ATCC 14821, *Rhodobacter sphaeroides* strain 2.4.1, and *Rhodopseudomonas palustris* strain GH were described before [28].

Growth of the bacteria and DNA isolation

The bradyrhizobia were grown in 50 ml Modified Arabinose Gluconate (MAG) broth [27] for the large-scale isolation of DNA purified by CsCl density centrifugation [19]. Concentrations of DNA in solution were measured spectrophotometrically at 260 nm by using a Gilford Response Spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH). MAG (10 ml) was used for small-scale DNA preparations using a Tissue and Blood DNA Extraction kit (Qiagen Inc., Chatsworth, CA).

Amplified Fragment Length Polymorphism (AFLP) analysis

The single restriction enzyme (*Pst*I) method described by Mueller et al. [18] was used to determine genome similarities among the *Bl. denitrificans* type strain (LMG8443) and 70 isolates originating from nodules of *A. indica*. This method was chosen because it was useful in demonstrating that soybean strains clustered according to serology [29]. Initial PCR amplifications were done by selecting for the single base with primer-G (the oligonucleotide with homology to the adapter sequences with a single selection base 'G' at the 3' end). The products were subsequently used in a second PCR reaction as templates by selecting for two bases using primer-GT, primer-GA or primer-GC. The resulting PCR products were separated on 1% horizontal agarose gels containing 0.5 µg/ml ethidium bromide and 0.5X TBE buffer (van Berkum, 1990). The gels were photographed using Kodak Tri-X pan film and the negatives were scanned into a computer for data analysis. The presence or absence of bands across lanes were scored using the software package Proscore version 2.39 (DNA Proscan, Nashville, TX). The banding patterns from the three primers were combined to produce a rectangular data matrix. The matrix was imported into NTSYSpc version 1.6 (Exeter Software, Setauket, NY) to derive simple matching coefficients across lanes and for SAHN clustering analysis using the unweighted pair group averages (UPGMA) method to generate a similarity tree.

PCR amplification for sequencing analysis

The primers 450 and 1440 [29] were used to amplify the ITS region [29] and are located in conserved regions of the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene. The PCR products generated with this primer pair also contained the Intervening region [6,11,13–15,21,22,35] of the 23S rRNA gene and permitted sequencing analysis of the 5' ends of the 23S rRNA genes using primers 1431, 1432, 1439 and 1440 [29]. The primer pair 1432 and 23SlowerB were used to amplify most of the 23S rRNA genes [33]. Primer 1432 is located upstream of the intervening region at bases 115–130 of the *B. japonicum* USDA 110 23S rRNA gene sequence GenBank accession Z35330. The reverse primer 23SlowerB is located in the 5S rRNA gene at bases 113–97 of the *B. japonicum* USDA 110 GenBank accession Z35330. PCR conditions were as those described before [33] using an annealing temperature of 52 °C in combination of a buffer containing 60 mM Tris–HCl, 15 mM (NH₄)₂SO₄, and 2.0 mM MgCl₂ at pH 9.0. Amplification reactions of partial regions of *pufM* used the primer set *pufM*.557F–*pufM*.750R [1] in

combination with a buffer containing 60 mM Tris–HCl, 15 mM (NH₄)₂SO₄, and 2.5 mM MgCl₂ at pH 8.5. PCR conditions were as those described for amplification of the 23S rRNA gene. The PCR products were purified using QIAquick Spin columns (Qiagen Inc., Chatsworth, CA). A Perkin Elmer 377 DNA Sequencer in combination with a Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA) was used for sequencing the purified PCR products as described by van Berkum et al. [30]. Sequencing primers have been described for the 23S rRNA gene [33] while the PCR primers for *pufM* also were used in sequencing reactions.

Analysis of the sequence data

The sequences were aligned using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, WI). Aligned sequences were checked manually and were edited with Genedoc [20]. UPGMA among differences in the number of nucleotides or derived amino acid residues were used to constructed trees using the Molecular Evolutionary Genetics Analysis (MEGA) package version 2.1 [12].

Nucleotide sequence accession numbers

The 23S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers [DQ011937](#), [DQ011938](#), [DQ011939](#), [DQ011940](#), [DQ011941](#), [DQ011942](#), [DQ011943](#) and [DQ011944](#) for USDA 4366, USDA 4407, USDA 4409, USDA 4415, USDA 4421, USDA 4424, USDA 4435 and USDA 4440, respectively. Those for the partial *pufM* sequence were assigned accession numbers [DQ017836](#) through [DQ017883](#) for USDA 4422, USDA 4426, USDA 4377, USDA 4379, USDA 4378, USDA 4365, USDA 4421, USDA 4364, USDA 4385, USDA 4404, USDA 4423, USDA 4424, USDA 4410, USDA 4425, USDA 4399, USDA 4418, USDA 4401, USDA 4428, LMG8443, USDA 4362(BTAi1), USDA 4373, USDA 4376, USDA 4405, USDA 4367, USDA 4368, USDA 4369, USDA 4370, USDA 4371, USDA 4375, USDA 4430, USDA 4417, USDA 4414, USDA 4392, USDA 4393, USDA 4402, USDA 4427, USDA 4391, USDA 4406, USDA 4429, USDA 4440, USDA 4403, *Rhodopseudomonas palustris* (strain GH), *Rhodobacter sphaeroides* (strain 2.4.1), *Rhodobacter capsulatus* (strain B10), *Methylobacterium extorquens* (ATCC 14718), *Methylobacterium extorquens* (ATCC 8457), *Methylobacterium rhodinum* (ATCC 14821) and *Methylobacterium radiotolerans* (ATCC 27329), respectively.

Electron microscopy

Bradyrhizobial cultures of strains recovered from nodules of *A. indica* were prepared for electron microscopy to examine cells for budding according to protocols used previously [28,32].

Phenotypic analyses

Carbon and nitrogen sources as substrates for growth were determined according to previously described methods [7,8,25]. Relative growth rates were measured turbidimetrically in MAG Broth described by van Berkum [27] at pH 7.0 in shaking cultures.

Results and discussion

The 16S rRNA gene and the internally transcribed spacer (ITS) sequences in the type strain for *Bl. denitrificans* reportedly are very similar with those of several bradyrhizobia isolated from nodules of *A. indica* [16,28,36]. One of the objectives of our study was to provide further evidence for the close genetic relationship between this type strain and these symbiotic bacteria. This was accomplished by sequencing the 23S rRNA genes [33] of several representative bradyrhizobial strains of *A. indica* and also by obtaining sequence comparisons of a small segment of the *pufM* gene that encodes the M subunit of the photosynthetic reaction center present in purple sulfur and purple nonsulfur phototrophs [1].

The 23S rRNA gene was chosen for this purpose because a number of sequences of this locus in α -Proteobacteria are available in the database [33]. The molecular sizes of products generated with primers 1432 and 23SlowerB that amplify a large portion of the 23S rRNA gene and the ITS region (between the 23S rRNA and 5S rRNA genes) were approximately 3.0 kb. Entire sequences for the 23S rRNA genes were obtained from analysis of two PCR products; the ITS region between the 16S rRNA gene and 23S rRNA gene obtained with PCR using primers 450 and 1440 [29], and sequences obtained for the remainder of the 23S rRNA gene.

Sequences from eight of the isolates were aligned with 23S rRNA gene sequences of LMG 8443 (*Bl. denitrificans*) and also with published sequences of other representative species within the α -Proteobacteria (Fig. 1). The alignment length was 3101 bp and average nucleotide frequencies were 20.3, 22.9, 26.0, and 30.7 for T, C, A, and G, respectively. Similarities of 23S rRNA gene sequences were derived by unweighted pair-group method analysis (UPGMA) of the number of nucleotide differences rather than through phylogenetic reconstruction because of the possibility of past recombination

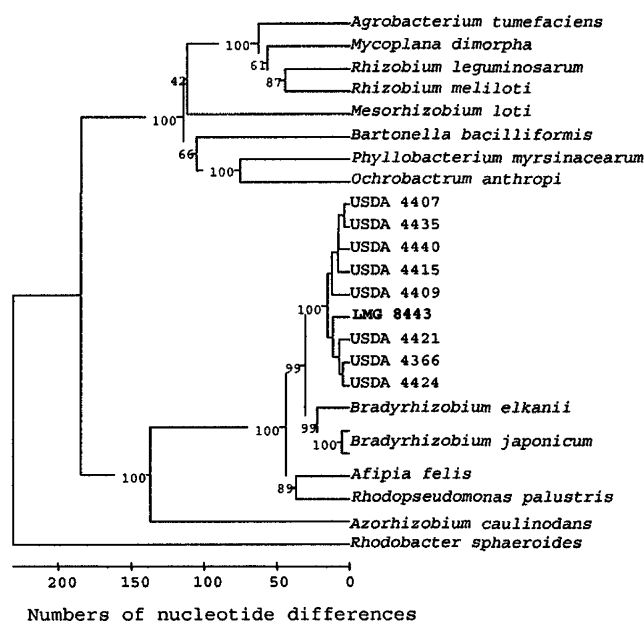


Fig. 1. Similarities of complete 23S rRNA gene sequences among select members of the α -Proteobacteria. The sequences were aligned using Pileup in the Wisconsin software Package and the resulting alignments were analyzed using Mega version 2.1 [12] after they had been checked manually using Genedoc [20]. Computations were made for the number of nucleotide differences among the aligned sequences, which was followed by tree construction using the Unweighted Pair Group Averages (UPGMA) algorithm. Bootstrap analysis to determine the confidence levels for the nodes used 500 permutations of the data set. To construct the tree, GenBank accession numbers AY244359 (*Rhizobium meliloti*), AY244361 (*Rhizobium leguminosarum*), AY244364 (*Mesorhizobium loti*), AY244367 (*Azorhizobium caulinodans*), AY244373 (*Agrobacterium tumefaciens*), AY244378 (*Mycoplasma dimorpha*), AY244379 (*Ochrobactrum anthropi*), AY244380 (*Phyllobacterium myrsinacearum*), U69638 (*Bradyrhizobium japonicum*), U35000 (*Bradyrhizobium elkanii*), AF338176 (*Blastobacter denitrificans*), AF338177 (*Afipia felis*), L39095 (*Bartonella bacilliformis*), X71839 (*Rhodopseudomonas palustris*), and X53855 (*Rhodobacter sphaeroides*) were used in conjunction with the sequences obtained in this work.

events among alleles of the *rrn* loci [33]. Such events of recombination between divergent alleles would be contradictory with the implied hierarchical relationships shown in a phylogenetic tree. The 23S rRNA gene sequences of the eight bradyrhizobial isolates from nodules of *A. indica* were most similar to each other and to the sequence of *Bl. denitrificans*. From the UPGMA tree it was evident that the isolates and *Bl. denitrificans* were placed together and aligned with two species within the genus *Bradyrhizobium* (Fig. 1). Placement of *Afipia felis* and *Rhodopseudomonas palustris* was on a proximal branch.

Another approach based on comparative analysis of partial *pufM* sequences was used to investigate the

genetic relationship between *Bl. denitrificans* and the bradyrhizobial isolates of *A. indica*. This approach was taken even though there is no evidence for the presence of bacterial photosynthesis genes in the genome of *Bl. denitrificans* as there is for the genomes of many bradyrhizobia of *A. indica*. Evidence for the presence of photosynthetic genes in the genome of *Bl. denitrificans* was obtained by PCR using primers to the conserved M subunit (*pufM*) of the photosynthetic reaction center present in purple sulfur and purple nonsulfur bacteria that yielded a 229 bp product [1]. PCR products of identical molecular size also were obtained using the isolates from *A. indica*, three species of *Methylobacterium*, two species of *Rhodobacter* and *Rhodopseudomonas palustris*. The nucleotide sequences of the *pufM* fragments of LMG 8443 and the isolates of *A. indica* were the most similar. The average difference was 9 nucleotides with a range from 0 to 22. By

comparison, the average nucleotide difference between LMG 8443 and the other phototrophs was 35 with a range from 31 to 43. Although there was nucleotide variation in the *pufM* sequence, *Bl. denitrificans* was placed within the group formed by the isolates originating from nodules of *A. indica* (Fig. 2A).

The deduced amino acid sequences of the partial PufM protein of *Bl. denitrificans* and the corresponding sequences obtained from the isolates of *A. indica* were identical (Fig. 2B). By comparison the average number of amino acid sequence differences between *Bl. denitrificans* and the other phototrophic species examined was 11, with the number of differences ranging from 9 to 14. Therefore, we concluded that the type strain for *Bl. denitrificans* possesses the gene coding for the reaction center *pufM*, and that this gene in the region examined codes for a protein that is identical with that of the bradyrhizobial isolates originating from *A. indica*.

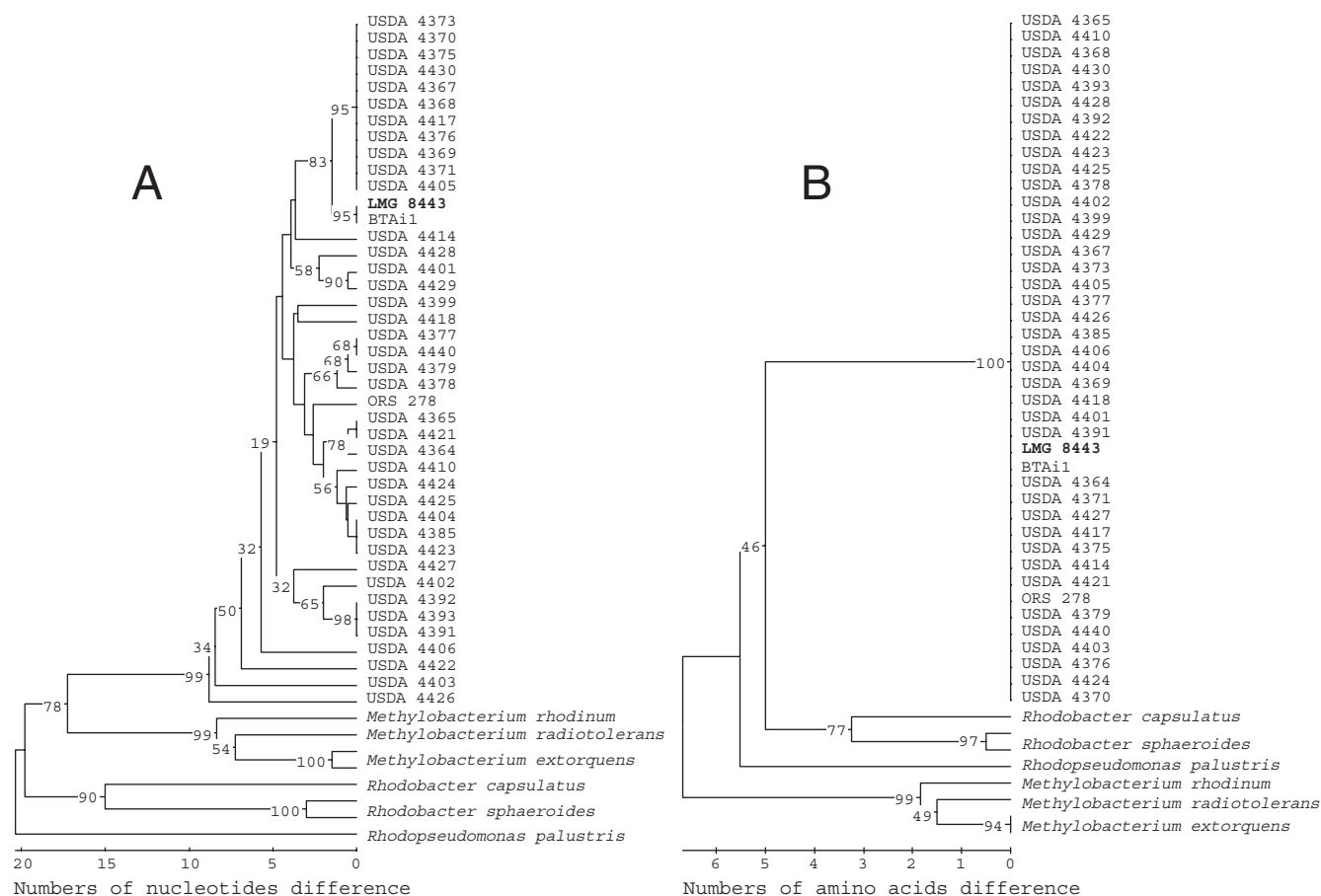


Fig. 2. Similarities of a partial segment of the *pufM* gene sequences among select members of phototrophic bacteria within the α -Proteobacteria. The sequences were aligned using Pileup in the Wisconsin software Package and the resulting alignments were analyzed using Mega version 2.1 [12] after they had been checked manually using Genedoc [20]. Computations were made for the number of nucleotide differences (A) or the number of deduced amino acid differences (B) among the aligned sequences, which was followed by tree construction using the Unweighted Pair Group Averages (UPGMA) algorithm. Bootstrap analysis to determine the confidence levels for the nodes used 500 permutations of the data set. To construct the tree, GenBank accession numbers AF182374 (bradyrhizobial strain ORS278), and AF195122 (*Rhodobacter sphaeroides*) were used in conjunction with the sequences obtained in this work.

Analysis of cellular morphology also was used to compare the type strain of *Bl. denitrificans* and the bradyrhizobia of *A. indica*. We chose to examine the ability of bacterial cells to propagate by budding since this was one of the primary criteria originally used to define the species *Bl. denitrificans* [8]. All of the isolates for which 23S rRNA gene sequences were determined were also examined for their ability to form budding cells in pure culture. Budding cells consistent with reported images of *Bl. denitrificans* were observed in all the cultures inspected (Fig. 3). Therefore, both the bradyrhizobial isolates originating from nodules of *A. indica* and the type strain of *Bl. denitrificans* share the ability to propagate by budding.

After having provided evidence for morphological and genetic similarity between the type strain of *Bl. denitrificans* and the isolates from nodules of *A. indica*, it was necessary to demonstrate that the collection of bacteria examined potentially represented a group that was diverse in phenotype and genotype. Genomic diversity was determined by AFLP analysis that is used

to examine variation in the location of nucleotide bases flanking specific restriction sites throughout the genome. In this case, variation in locations of *Pst*I in conjunction with changes in “GT”, “GA” and “GC” as flanking bases were examined. The analysis of 71 genomes, including the type strain of *Bl. denitrificans*, yielded 5112 data points that were used to calculate simple matching coefficients for clustering analysis. From a similarity tree derived from the clustering analysis it was evident that genomic variation existed among all 71 members of the collection that was examined (Fig. 4). Therefore, genetic diversity among isolates and *Bl. denitrificans* was established.

Phenotypic diversity in the collection was examined by the ability of the type strain of *Bl. denitrificans* and seven isolates of *A. indica* to utilize a range of different carbon (1.0 g/l) and nitrogen sources for growth, and also by determining the ability to grow in broth culture at 41 °C.

Although our results with the type strain of *Bl. denitrificans* were similar to those described by Hirsch

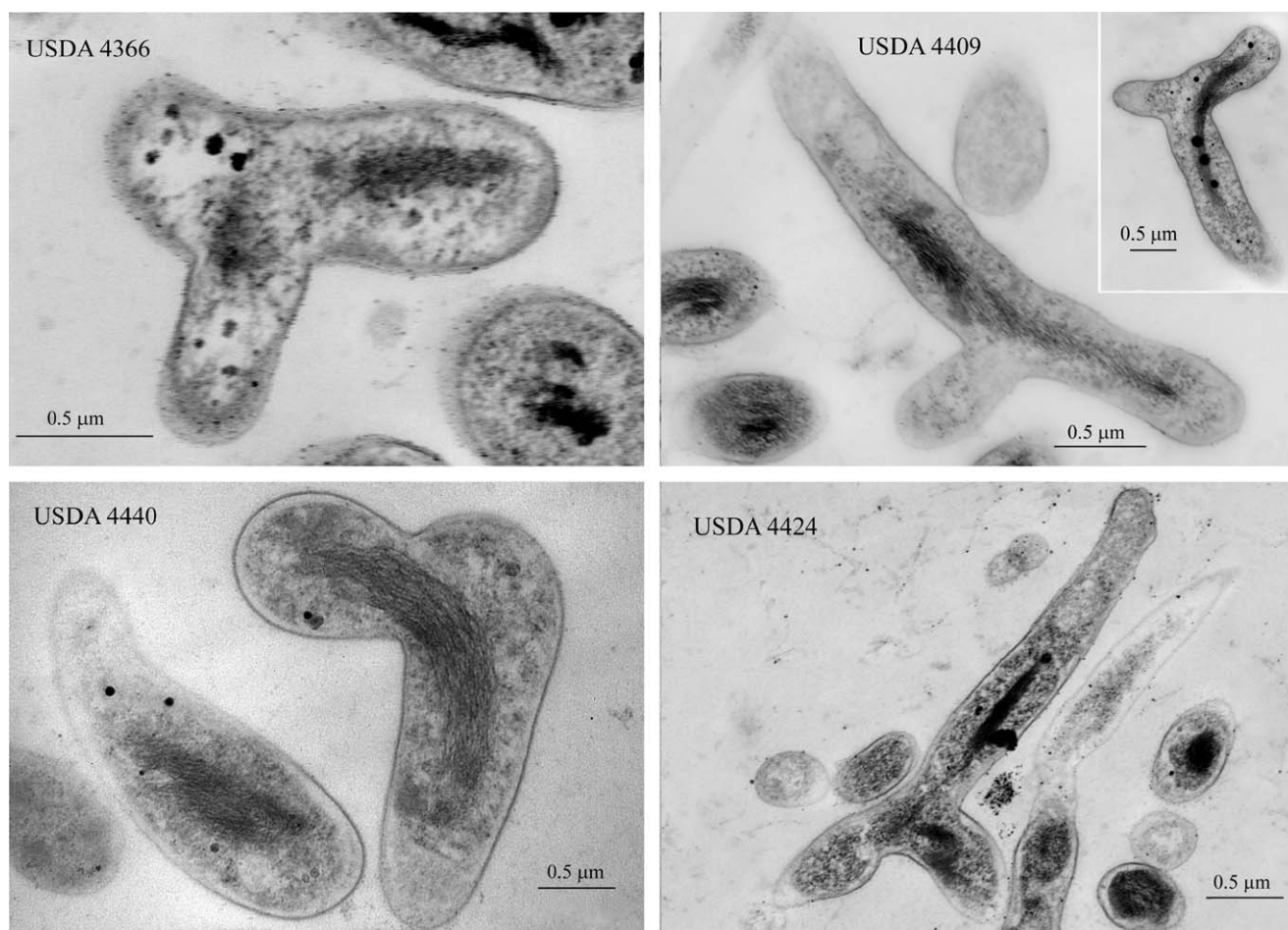


Fig. 3. Electron micrographs of several bradyrhizobial isolates from nodules of *Aeschynomene indica* demonstrating cell propagation by budding.

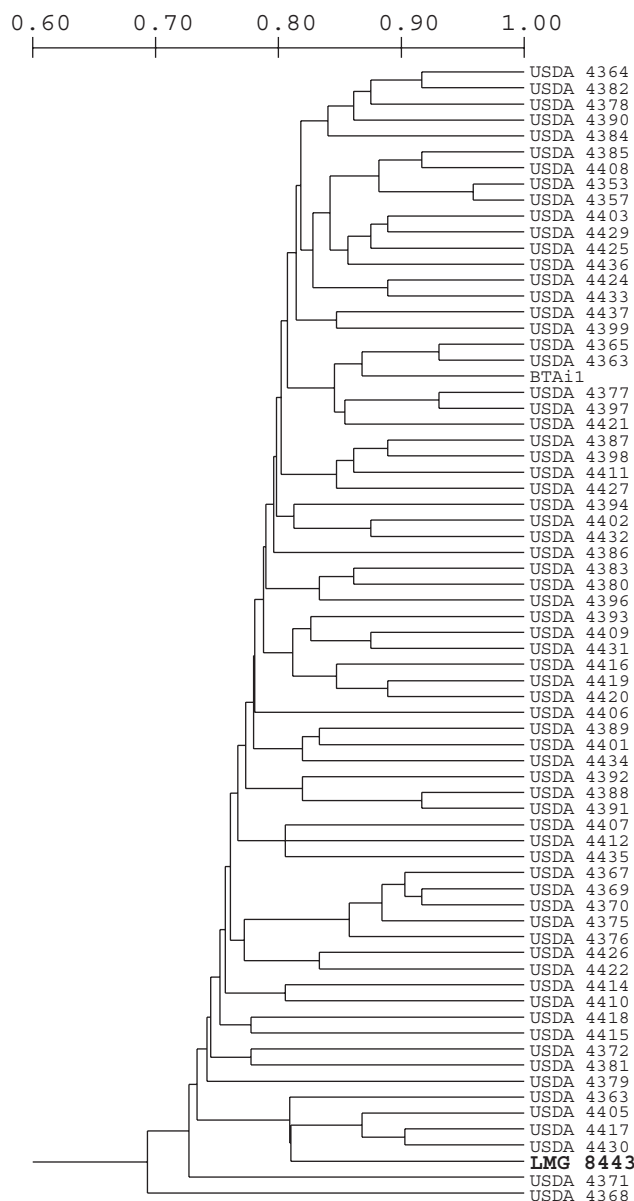


Fig. 4. Genomic variation among bradyrhizobial isolates from nodules of *Aeschynomene indica* and the type strain for *Blastobacter denitrificans* (LMG 8443) determined by Amplified Fragment Length Polymorphism. The procedures of analysis and the construction of the similarity tree were as described by van Berkum and Fuhrmann [29].

and Mueller [8], there were some notable exceptions. In our hands *Bl. denitrificans* (LMG 8443) utilized citric acid, while it was unable to utilize formamide and formic acid as carbon sources, and urea and methylamine HCl as nitrogen sources. In accordance with Hirsch and Mueller [8], who considered the ability to utilize urea as being a key diagnostic criterion for the species *Bl. denitrificans*, five of the seven bradyrhizobial isolates from *A. indica* nodules that were examined did utilize urea.

Of the seven isolates examined four were unable to grow at 41 °C, while the remaining three and the type strain for *Bl. denitrificans* grew at this temperature. One isolate, BTAi1, and the type strain for *Bl. denitrificans* grew optimally at 41 °C.

Description of *Bradyrhizobium denitrificans* comb. nov.

The description of the type strain LMG 8443 of *B. denitrificans* (de.ni.tri'fi.cans L. prep. de away from; L. n. *nitrum* soda; M. L. n. *nitrum* nitrate; M. L. v. *denitrifico* denitrify; M. L. part. adj. *denitrificans* denitrifying) is the same as the genus [10] and species description [8] and includes the following:

Cells rod-shaped with rounded poles. Buds rod-shaped and originating sub-polarly (Fig. 3). Cells are noncapsulated, gram-negative, not acid-fast, do not form spores or cysts, $0.6\text{--}0.8 \times 1.5\text{--}2.3\text{ }\mu\text{m}$. Grow in liquid media as turbidity. Colonies glistening, raised and round with entire edges, and may form pink or orange pigments with light exposure or in the dark. Grow well on dilute media at 30 °C, some strains tolerate 41 °C. Chemoorganotrophic, they utilize a range of carbohydrates including: α -oxoglutarate, fructose, galactose, glucose, glutamate, glycerol, lactose, mannitol, pyruvate, ribose, and succinic acid. Some strains also utilize acetate, citrate, ethanol (0.4% v/v), formate, formamide, fumarate, glycine, glucuronic acid lactone, histidine, leucine, malate, maltose, methanol (0.5% v/v), *N*-acetyl glucosamine, proline, sucrose, and tartrate as carbon sources. Carbon sources that are not utilized include: alanine, arginine, aspartate, caproate, cellobiose, dextrin, indole, inulin, lactate, phenylalanine, phthalate, propionate, salicin, serine, or starch. Pigmented strains are capable of aerobic heterotrophic photosynthesis growth under light. Ammonium salts, Bacto-peptone, casein hydrolysate, and yeast extract are all utilized as nitrogen sources. Most strains can utilize nitrate as a sole nitrogen source, and most are capable of dissimilatory nitrate reduction. Nitrite is not utilized as a nitrogen source. Some strains are capable of utilizing methylamine HCl and urea as sole nitrogen sources. Strains are able to invade roots and stems of some species of *Aeschynomene* and elicit the production of root nodules, where they participate in endosymbiotic nitrogen fixation.

Acknowledgments

The authors express their appreciation to Charlie Murphy for electron microscopy, and to Patrick Elia and K. Lee Nash for technical assistance.

References

- [1] L. Achenbach, J. Carey, M.T. Madigan, Photosynthetic and phylogenetic primers for detection of anoxygenic phototrophs in natural environments, *Appl. Environ. Microbiol.* 67 (2001) 2922–2926.
- [2] D. Alazard, Stem and root nodulation in *Aeschynomene* spp, *Appl. Environ. Microbiol.* 50 (1985) 832–834.
- [3] A.R.J. Eaglesham, A.A. Szalay, Aerial stem nodules on *Aeschynomene* spp, *Plant Sci. Lett.* 29 (1983) 265–282.
- [4] A.R.J. Eaglesham, J.M. Ellis, W.R. Evans, D.E. Fleischmann, M. Hungria, R.W.F. Hardy, The first photosynthetic N₂-fixing *Rhizobium*: characteristics, In: P.M. Gresshoff, L.E. Roth, G. Stacey, W.E. Newton (Eds.), *Nitrogen Fixation: Achievements and Objectives*, Chapman & Hall, New York, 1990, pp. 805–811.
- [5] W.R. Evans, D.E. Fleischmann, H.E. Calvert, P.V. Pyati, G.M. Alter, N.S. Subba Rao, Bacteriochlorophyll and photosynthetic reaction centers in *Rhizobium* strain BTAi1, *Appl. Environ. Microbiol.* 56 (1990) 3445–3449.
- [6] E. Evgueniev-Hackenberg, S. Selenska-Pobell, Variability of the 5'-end of the large subunit rDNA and the presence of a new short class of rRNA in *Rhizobiaceae*, *Lett. Appl. Microbiol.* 21 (1995) 402–405.
- [7] P. Gerhardt, *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington, DC, 1994.
- [8] P. Hirsch, M. Müller, *Blastobacter aggregatus* sp. nov., *Blastobacter capsulatus* sp. nov., and *Blastobacter denitrificans* sp. nov., new budding bacteria from fresh water habitats, *Syst. Appl. Microbiol.* 6 (1985) 281–286.
- [9] P. Hugenholtz, E. Stackebrandt, J.A. Fuerst, A phylogenetic analysis of the genus *Blastobacter* with a view to its future reclassification, *Syst. Appl. Microbiol.* 17 (1994) 51–57.
- [10] D.C. Jordan, Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing root nodule bacteria from leguminous plants, *Int. J. Syst. Bacteriol.* 32 (1982) 136–139.
- [11] E. Kordes, S. Jock, J. Fritsch, F. Bosch, G. Klug, Cloning of a gene involved in rRNA precursor processing and 23S rRNA cleavage in *Rhodobacter capsulatus*, *J. Bacteriol.* 176 (1994) 1121–1127.
- [12] S. Kumar, K. Tamura, M. Nei, MEGA: Molecular Evolutionary Genetics Analysis, 1.01 ed., The Pennsylvania State University, University Park, PA, 1993.
- [13] T.G. Lessie, The atypical ribosomal RNA complement of *Rhodopseudomonas sphaeroides*, *J. Gen. Microbiol.* 39 (1965) 311–320.
- [14] M.L. Mackay, B. Zablen, C.R. Woese, W.F. Doolittle, Homologies in processing and sequence between the 23S ribosomal ribonucleic acids of *Paracoccus denitrificans* and *Rhodopseudomonas sphaeroides*, *Arch. Microbiol.* 123 (1979) 165–172.
- [15] B. Marrs, S. Kaplan, 23S precursor ribosomal RNA of *Rhodopseudomonas sphaeroides*, *J. Mol. Biol.* 49 (1970) 297–317.
- [16] F. Molouba, J. Lorquin, A. Willems, B. Hoste, E. Giraud, B. Druyfus, M. Gillis, P. de Lajudie, C. Masson-Boivin, Photosynthetic bradyrhizobia from *Aeschynomene* spp. are specific to stem-nodulated species and form a separate 16S ribosomal DNA restriction fragment length group, *Appl. Environ. Microbiol.* 65 (1999) 3084–3094.
- [17] W.E.C. Moore, L.V.H. Moore, Index of the Bacterial and Yeast Nomenclatural Changes, American Society for Microbiology, Washington, DC, 1992.
- [18] U.G. Mueller, S.E. Lipari, M.G. Milgroom, Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*, *Mol. Ecol.* 5 (1996) 119–122.
- [19] R.B. Navarro, A.A.T. Vargas, E.C. Schröder, P. van Berkum, Uptake hydrogenase (Hup) in common bean (*Phaseolus vulgaris*) symbioses, *Appl. Environ. Microbiol.* 59 (1993) 4161–4165.
- [20] K.B. Nicholas, H.B. Nicholas, Genedoc: a tool for editing and annotating multiple sequence alignments, Multiple Sequence Alignment Editor and Shading Utility, 2.6.001. Distributed by the authors, 1997.
- [21] W. Schuch, U.E. Loening, The ribosomal ribonucleic acid of *Agrobacterium tumefaciens*, *Biochem. J.* 149 (1975) 17–22.
- [22] S. Selenska-Pobell, E. Evgueniev-Hackenberg, Fragmentations of the large-subunit rRNA in the family *Rhizobiaceae*, *J. Bacteriol.* 177 (1995) 6993–6998.
- [23] L.I. Sly, Emendation of the genus *Blastobacter* Zavarzin 1961 and description of *Blastobacter natatorius* sp. Nov., *Int. J. Syst. Bacteriol.* 47 (1985) 211–213.
- [24] L.I. Sly, M.M. Cahill, Transfer of *Blastobacter natatorius* (Sly 1985) to the genus *Blastomonas* gen. nov. As *Blastomonas natatoria* comb. Nov., *Int. J. Syst. Bacteriol.* 47 (1997) 566–568.
- [25] J.T. Staley, Budding bacteria of the *Pasteuria-Blastobacter* group, *Can. J. Microbiol.* 19 (1973) 609–614.
- [26] Y.A. Trotenko, N.V. Doronina, P. Hirsch, Genus *Blastobacter*, In: J.T. Staley, M.P. Bryant, N. Pfennig, J.G. Holt (Eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 3, The Williams & Wilkins Co., Baltimore, MD, 1989, pp. 1963–1968.
- [27] P. van Berkum, Evidence for a third uptake hydrogenase phenotype among the soybean bradyrhizobia, *Appl. Environ. Microbiol.* 56 (1990) 3835–3841.
- [28] P. van Berkum, B.D. Eardly, The aquatic budding bacterium *Blastobacter denitrificans* is a nitrogen-fixing symbiont of *Aeschynomene indica*, *Appl. Environ. Microbiol.* 68 (2002) 1132–1136.
- [29] P. van Berkum, J.J. Fuhrmann, Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and Internally Transcribed Spacer region sequence divergence, *Int. J. Syst. Evol. Microbiol.* 50 (2000) 2165–2172.
- [30] P. van Berkum, D. Beyene, B.D. Eardly, Phylogenetic relationships among *Rhizobium* species nodulating the common bean *Phaseolus vulgaris* L., *Int. J. Bacteriol.* 46 (1996) 240–244.
- [31] P. van Berkum, R.E. Tully, D.L. Keister, Nonpigmented and bacteriochlorophyll-containing bradyrhizobia isolated from *Aeschynomene indica*, *Appl. Environ. Microbiol.* 61 (1995) 623–629.
- [32] P. van Berkum, D. Beyene, G. Bao, T.S. Campbell, B.D. Eardly, *Rhizobium mongolense* sp. nov. is one of three

- rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.)Ledebour], Int. J. Syst. Bacteriol. 48 (1998) 13–22.
- [33] P. van Berkum, Z. Terefework, L. Paulin, S. Suomalainen, K. Lindstrom, B.D. Eardly, Discordant phylogenies with the *rrn* loci of rhizobia, J. Bacteriol. 185 (2003) 2988–2998.
- [34] A. Willems, M.D. Collins, Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences, Int. J. Syst. Bacteriol. 43 (1993) 305–313.
- [35] M.E. Winkler, Ribosomal ribonucleic acid isolated from *Salmonella typhimurium*: absence of the intact 23S species, J. Bacteriol. 139 (1979) 842–849.
- [36] F.Y.K. Wong, E. Stackebrandt, J.K. Ladha, D.E. Fleischman, R.A. Date, J.A. Fuerst, Phylogenetic analysis of *Bradyrhizobium japonicum* and photosynthetic stem-nodulating bacteria from *Aeschynomene* species grown in separated geographical regions, Appl. Environ. Microbiol. 60 (1994) 940–946.
- [37] J.P.W. Young, H.L. Downer, B.D. Eardly, Phylogeny of the phototrophic Rhizobium strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment, J. Bacteriol. 173 (1991) 2271–2277.
- [38] G.A. Zavarzin, Budding bacteria, Mikrobiologiya 30 (1961) 952–975.